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Human Papillomavirus Antigen and DNA in Human Oral Cancer Tissues

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INTRODUCTION: Compelling evidence, obtained through investigations during the past decade, suggests the association of specific human papillomaviruses (HPV) with a wide range of epithelial malignancies. Several studies have strongly implicated HPVs in benign, premalignant and malignant lesions of the anogenital tract (1). DNA of specific HPV types is detectable in about 90% of cervical carcinomas, the most prevalent being HPV-16 DNA in about 64% of the cervical cancers and HPV-18 DNA in 3% (2). Epidemiological studies have attributed Squamous Cell Carcinoma of the oral cavity to the abuse of tobacco and alcohol. However, a multifactorial aetiology for this disease is more probable. The possible aetiological role for HPV in cervical carcinoma, the obvious oncogenic potential of certain HPV types and the close similarity between oral and genital mucosa justify a search for a causative role for HPV in oral squamous cell carcinoma (3). Besides, a few studies undertaken abroad have indicated an association between HPV and oral cancer (3). During the present investigation, we studied oral squamous carcinoma tissue samples for the presence of papillomavirus structural antigens. We also looked for the presence of HPV-16 and HPV-18 DNA sequences in oral cancer tissue DNA samples using DNA blot hybridisation analysis.

MATERIAL AND METHODS:

Detection of Papillomavirus Structural Antigens: Tissue samples from 54 histopathologically proved oral squamous epithelial carcinomas and 20 normal oral tissue samples were included in the study. About 6 um thick sections of formalin-fixed, paraffin-embeded tissue samples were processed for the immunohistochemical demonstration of papillomavirus structural antigens by peroxidase-antiperoxidase (PAP) technique. The 'DAKO PAP KIT' (Dakopatts, USA), which detect papillomavirus structural antigen, was used, and the procedure supplied by the manufacturer was followed.

Detection of HPV DNA Sequences :

DNA Extraction: DNA samples from biopsy and surgery specimens from histopathologically confirmed oral squamous carcinoma cases and normal oral mucosa samples, obtained from fresh autopsy specimens, were used in DNA hybridisation studies. Tissue DNA was extracted by the standard

phenol-chloroform-isoamyl alcohol method. Briefly, the samples were homogenized in Tris-EDTA buffer, 0.5% final concentration of SDS and 100 ug/ml Proteinase K were added to the hemogenate and incubated for 3 hr to overnight at 37°C. The digest was then extracted with wate-saturated phenol. The aqueous layer was collected and re-extracted once with phenol-chloroform-isoamyl alcohol followed by chloroform-isoamyl alcohol. DNA was precipitated cut with 1/10 volume of chilled 3 M sodium acetate and 2.5 volume of absolute ethanol. The DNA pellet was washed in 70% ethanol, freeze-dried and resuspended in tris-EDTA buffer (2).

Probes: HPV-16 and 18 DNA, (8 kb) cloned in pBR 322, were used for the preparation of probes in this study. The vectors containing the HPV inserts were digested with appropriate restriction enzymes and electrophoresed in a 0.7% low melting point agarose gel. The HPV DNA bands were cut out and DNA extracted from the gel by phenol extraction or electro elution. The vector-free HPV inserts were labelled with alpha 32P dATP by random priming, using 'Megaprime DNA labelling systems' (Amsterdam, England). The specific activity of the probes used was greater than $1\text{-}2 \times 10^8$ cpm/ug DNA.

Dot Blotting: 8 to 10 ug of the DNA from oral cancer and normal tissue samples were denatured at 65°C for 30 minutes, snap-chilled on ice and blotted on to a nylon membrane (Hybond-N; Amersham, U.K.) using a dot blot apparatus. Dots of positive and negative control DNA were always incorporated. The membrane was baked at 80°C for 2 hours.

Southern Blotting: About 10 ug of DNA from each sample was digested with Bam HI or Pst I, and electrophoresed in 1% agarose gel in TAE buffer (2 M Tris, 0.05 M EDTA, adjusted to pH 8 with glacial acetic acid) containing ethedium bromide (1 ul of 10 mg/ml solution /60 ml agarose solution). Electrophoresis was conducted at 1 V/cm of gel for 16-20 hr, and the gel was pohotographed under a UV transilluminator. The gel was then treated with denaturing buffer (1.5 M NaCl + 0.5 M NaOH) and then neutralised with neutralisation buffer (0.5 M Tris-HCl + 1.5 M NaCl, pH 7.5). The DNA bands were transferred overnight on to Hybond-N nylon membrane by capillary Southern blotting. The membrane was washed with 6 x SSC (0.09 M trisodium citrate, 0.9 M NaCl; pH 7.0), airdried and barked at 80°C for 2 hrs. 10 pg of viral DNA, approximately equivalent to one viral genome per cell, that was incorporated into the blot served as positive control.

Hybridisation: The blots were prehybridised in prehybridisation solution (50% formamide, 5x SSC, 0.02% Denhardt's solution, 50 ug/ml herring sperm DNA, 50 mM trisodium phosphate and 1% SDS) for 2 hr at 42°C. Hybridisation was carried out for 16 to 20 hr under stringent conditions, at 42°C in the hybridisation solution containing 50% formamide, 5 x SSC, 0.02% Denhardt's solution, 50 ug/ml denatured herring sperm DNA, 1% SDS, 10% Dextran sulphate, 50 mM trisodium phosphate and the radiolabelled probe. After hybridisation, the membranes were subjected to stringent washes and then autoradiographed.

RESULTS AND DISCUSSION: 54 oral cancer tissue samples were studied for papillomavirus structural antigens by PAP technique. 28 samples (52%) were positive. The nuclear staining was mainly confined to superficial layers of the squamous epithelium. The antigen distribution was usually in a focal

and uniform pattern, but in a few cases the antigen positive nuclei were observed in diffuse patches. None of the 20 normal samples studied were positive for the viral antigens.

52 DNA samples from oral squamous carcinoma were studied for HPV-16 and 18 DNA sequences by dot blot hybridisation. 31 (60%) samples were positive for HPV-16 and 4 (8%) for HPV-18. Two samples were positive for both HPV-16 and HPV-18. None of the 18 DNA samples from normal oral mucosa, studied for both HPV-16 and 18, was found to be positive for the viral DNA.

Southern blot analysis was employed for 34 DNA samples from oral squamous carcinoma cases. HPV-16 DNA was noted in 18 (53%) samples and HPV-18 DNA in 3 (9%) samples. One DNA sample harboured both HPV-16 and HPV-18 DNA sequences. 15 samples from normal oral mucosa were found to contain no viral DNA. The 8 kb authentic Bam HI band lighted up in the autoradiograms of all the HPV-16 DNA positive samples. Characteristic Pst I restriction fragments (2.8, 1.9, 1.6, 1 and 0.5 kb) were observed in the HPV-16 positive cases studied. However, altered intensity or size and addition or absence of the restriction fragments, indicating difference in their copy number and minor possible viral genomic rearrangements could be noted in a few samples. Majority of the positive samples were found to contain a rather low level of the viral genomes. 4 samples contained very high copy number of HPV-16 DNA.

Initial indication of an association between oral carcinoma, and HPV originated from the demonstration of papillomavirus structural antigen in histological sections of the cancer tissues (4). Using DNA hybridisation technique and polymerase chain reaction, a few subsequent studies demonstrated the presence of HPV DNA. De Villiers et al (5) reported HPV-16 and HPV-2 DNA in tongue carcinoma. Two in situ DNA hybridisation studies (6,7) have noted HPV-16 and 18 DNA in oral squamous cell carcinomas. Studies made by Chang et al (8) detected HPV-16 DNA in 76% of the oral carcinomas in a group of Taiwanese patients. HPV-18, HPV-4 and a variant of HPV-16 were detected in oral squamous cell carcinoma by Yeudall and Campo (9). Maitland et al (10) demonstrated a variant of HPV-16 in 46% of the oral cncer biopsies and in 5/12 normal oral mucosa samples. Using polymerase chain rection Maitland et al (11) later demonstrated HPV-16 DNA sequences in oral cancer biopsies and in the cell lines derived from them.

In the present study, we could demonstrate papillomavirus structural antigens and HPV-16 and HPV-18 DNA in a considerable number of oral squamous carcinoma tissue samples but not in normal oral mucosa. The results of our study is in general agreement with those of previous studies that have suggested a more than accidental association of HPVs with oral squamous carcinoma. Recent studies (3,12) have shown the cell transformation potential of specific HPV types in presence of cofactors and it has been shown that E6 and E7 proteins of high risk HPVs can interact with human retinoblastoma gene product (pRb) and the tumor suppressor gene product, p53, respectively in cell transformation. The high frequency occurrence of high risk HPVs in oral cancer and their known cell transofrmation potential suggest a possible aetiological role for HPV in oral neoplasia. Epidemiological studies have noted betal quid chewing and other forms of abuse of tobacco and consumption

of alcohol to be the major aetiological factors in oral carcinoma. It may be that HPVs, in Synergism with chemical carcinogens and/or other cofactors, play some role in carcinogenesis.

REFERENCES:

1. Zur Hausen H: Papillomaviruses as carcinomaviruses. In Klein G (ed.); Advances in Viral Oncology, Vol.8, New York. Raven, pp. 1-26, 1989.

2. Das BC, Sharma JK, Gopalkrishna V, Das DK, Singh V, Gissmann L, Zur Hausen H, Luthra UK: J Med Virol; 36:239-245, 1992.

3. Yeudall WA: Oral Oncol, Eur J Cancer, 28B: 61-66, 1992.

4. Syrjanen K, Syrjanen S, Lamberg M, Pyrhonen S, Nuutinen J: Int J Oral Surg, 12:418-424, 1983.

5. De Villiers EM, Weidaner Otto H, Zur Hausen H: Int J Cancer, 36:575-578, 1985.

6. Milde K, Loning T: J Oral Pathol, 15:292-296, 1986.

7. Syrjanen S, Syrjanen KJ, Happonen RP: J Oral Pathol Med, 17:273-278, 1988.

8. Chang KW, Chang CS, Lai KS, Chou MJ, Choo KB; J Med Virol; 28:57-61, 1989.

9. Maitland NJ, Cox MF, Lynas C, Prime SS, Meanwell CA, Scully C: Br J Cancer, 56:245-250, 1987.

10. Yeudall WA, Campo MS: J Gen Virol, 72:173-176; 1991.

11. Maitland NJ, Bromide T, Cox MF, Crane IJ, Prime SS, Scully C: Br J Cancer, 59: 698-703, 1989.

12. Howley PM: Cancer Res. (Suppl.), 51:5019s-5022s, 1991.